Oxalic acid degradation by a novel fungal oxalate oxidase from Abortiporus biennis

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Oxalate oxidase was identified in mycelial extracts of a basidiomycete Abortiporus biennis strain. Intracellular enzyme activity was detected only after prior lowering of the pH value of the fungal cultures by using oxalic or hydrochloric acids. This enzyme was purified using size exclusion chromatography (Sephadex G-25) and ion-exchange chromatography (DEAE-Sepharose). This enzyme exhibited optimum activity at pH 2 when incubated at 40°C, and the optimum temperature was established at 60°C. Among the tested organic acids, this enzyme exhibited specificity only towards oxalic acid. Molecular mass was calculated as 58 kDa. The values of Kₘ for oxalate and Vₘₐₓ for the enzyme reaction were 0.015 M and 30 mmol min⁻¹, respectively.

Key words: oxalate oxidase, oxalic acid, Abortiporus biennis

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INTRODUCTION

Abortiporus biennis is a ligninolytic basidiomycete fungus belonging to the ecological group of white rot fungi that are very efficient natural wood degraders. These fungi possess a unique system for efficient degradation of a lignin polymer which constitutes the major carbon source on Earth. The main constituents of this system are extracellular enzymes, like laccase and different peroxidases – manganese-dependent peroxidase (MnP), lignin peroxidase (LiP), and versatile peroxidase (VP) (Jarosz-Wilkołazka et al., 2008; Polak & Jarosz-Wilkołazka, 2012). Enzymatic degradation of lignin is supported in multiple ways by the action of low-molecular-weight compounds (Kaneko et al., 2005), and among them, a very important role is played by oxalic acid. This dicarboxylic acid is a predominant organic acid secreted by many fungi and has several ecological roles, e.g. facilitation of the MnP action by chelating unstable Mn²⁺ ions or buffering pH values outside the hyphae (Hofrichter, 2002). The biosynthesis of oxalic acid in fungi most likely implicates the tricarboxylic acid cycle, involving the hydrolytic cleavage of oxaloacetate to acetate and oxalate, or the glyoxylate cycle involving oxidation of glyoxylate to oxalate (Mäkelä et al., 2010). There is diversity in secretion of oxalic acid among various ecological groups of wood degrading fungi. Brown rot fungi generally secrete oxalic acid in higher amounts than white rot fungi (Hastrup et al., 2012; Mäkelä et al., 2010). This can be due to the fact that white rot fungi are able to decompose oxalic acid via the action of oxalate decarboxylase (EC 4.1.1.2), which converts oxalate to formic acid and carbon dioxide (Mäkelä et al., 2002). The degradation of oxalate via action of oxalate oxidase (EC 1.2.3.4), described in our study, is atypical for fungi and was found predominantly in higher plants. The best characterised oxalate oxidase originates from cereal plants (Dunwell, 2000). Currently, only three oxalate oxidases of basidiomycete fungi have been described - an enzyme from Tilletia controversa (Vaisey et al., 1961), the best characterised so far enzyme from Ceriporiopsis subvermispora (Aguilar et al., 1999), and an enzyme produced by Abortiporus biennis (Grąz et al., 2009). The enzyme from C. subvermispora was also expressed in Pichia pastoris and characterised as Mn(II)-containing bicuspid protein sharing homology with bicuspid microbial oxalate decarboxylase (Moussatche et al., 2011). Oxalate oxidase decomposes oxalic acid to carbon dioxide and hydrogen peroxide. Hydrogen peroxide is in turn the key component of the catalytic cycle of all peroxidases and can also be generated by fungal feedback-type enzymes, like glucose-1-oxidase (EC 1.1.3.4), aryl alcohol oxidases (EC 1.1.3.7), and pyranose-2-oxidase (EC 1.1.3.10) (Leonowicz et al., 1999). Oxalic acid also plays a significant role in biological and geochemical processes in the soil (Gadd, 2007). For example, oxalate has a large effect on the availability of phosphorous and calcium, and is associated with weathering of soil minerals and precipitation of insoluble metal oxalates (Jarosz-Wilkołazka & Grąz, 2006, Jarosz-Wilkołazka et al., 2006, Graż et al., 2009). Oxalic acid is a known toxic compound associated with plant pathogenic fungal strains, such as Sclerotinia sclerotiorum (Hegedus & Rimmer, 2005). The level of oxalic acid is also an important factor in many technologies applied in the industry, such as the paper industry or brewing processes (Dunwell et al., 2000, Sjöde et al., 2008).

In this study, we examined factors influencing the induction of the oxalate oxidase activity in cultures of white rot fungus Abortiporus biennis and characterised selected properties of the novel fungal oxalate oxidase (OXOAb).

MATERIALS AND METHODS

The organism and growth conditions. Abortiporus biennis was obtained from the Fungal Collection of the Biochemistry Department, Maria Curie-Skłodowska University, Lublin, Poland. Stock cultures were maintained on 2% malt extract agar (MEA) slants at 4°C. The inoculum was pre-cultured on 2% MEA for one week.

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Abbreviations: OXO, oxalate oxidase; OXOAb, oxalate oxidase from Abortiporus biennis
week at 25°C. The experiments were performed in Erlemeyer flasks using liquid medium containing glucose (10 g L⁻¹) and potato extract (4 g L⁻¹). The inoculated flasks were incubated at 25°C with a rotation mode (160 rpm). After 7 days of cultivation, OXOAb production by the A. biennis strain was induced by addition of oxalic acid, sodium oxalate, or hydrochloric acid (to the final concentration of 10 mM) to the growth media. After 24 hours, the mycelium was harvested and homogenised. The mycelial extract was centrifuged, assayed for the OXOAb activity, and frozen for further analysis.

Oxalate oxidase activity (OXOAb) assay (Aguilar et al., 1999). The standard assay of this enzyme was based on the measurement of enzymatically generated hydrogen peroxide. The reaction mixture contained 0.3 mL of 20 mM oxalic acid in 0.05 M succinate buffer, pH 3.5, and 0.2 mL of the enzyme. After incubation of the reaction mixture for 15 min at 40°C, 0.45 mL of 0.2 mM phenol red solution and 0.05 mL of horseradish peroxidase (6.25 U) in 0.05 M succinate buffer, pH 3.5, were added. Following incubation at 30°C for 15 min, 0.1 mL of 5 M NaOH was added and the absorbance at 610 nm was measured. A standard curve was used to calculate the amount of H₂O₂ (prepared in 0.05 M succinate buffer) generated during the OXOAb reaction. One enzyme unit was defined as the amount of enzyme required to produce 1 mmol of H₂O₂ per minute, under standard assay conditions.

To determine the optimum pH value for OXOAb activity, the enzyme was incubated with oxalic acid in 0.05 M succinate buffer of pH 2, 2.5, 3, 3.5, and 4. The optimum temperature for OXOAb activity was determined using succinate buffer pH 3.5 and incubation at 30°C, 40°C, 50°C, 60°C, 70°C, and 80°C. To determine the substrate specificity of OXOAb, the standard activity assay was performed using citric, malic, malonic, glycolic, glioxalic, succinic, and acetic acids at 2 mM and 20 mM concentrations at pH 3.5. The Kₘ and Vₘₐₓ values were determined using standard conditions of the reaction.

Determination of protein concentration. Protein concentration was determined using the Bradford method (1976) and bovine serum albumin was used as a standard.

Determination of hydrogen peroxide concentration. The chemiluminescence method based on the Co (II)-catalysed oxidation of luminol by H₂O₂ was applied according to Perez and Rubio (2006).

Determination of organic acid concentration. The concentration of organic acids in the cultures of A. biennis was monitored by capillary electrophoresis using an Agilent 7100 Capillary Electrophoresis System equipped with a UV-Vis detector. The separation was carried out using a fused silica capillary 50 μm ID with a 50 cm length to the detection window. The voltage applied was −25 kV and the capillary temperature was maintained at 15°C. Samples were injected hydrodynamically for 5 s (50 mbar) and organic acid was detected by indirect UV-detection at a wavelength of 350 nm (bandwidth 20 nm) and a reference wavelength of 230 nm (bandwidth 10 nm). The buffer solution was freshly prepared every day by dissolving phthalic acid (5 mM), cetyltrimethylammonium bromide (CTAB, 0.26 mM), and methanol (0.5% v/v) in MilliQ water (Chen et al., 1997). Peak identification was done by spiking with commercially available formic and oxalic acids.

Enzyme purification. Gel filtration on Sephadex G-25. The crude extract obtained after homogenization and centrifugation of induced mycelium of A. biennis was loaded onto a Sephadex G-25 column (2.5 × 30 cm) previously equilibrated with 20 mM phosphate buffer pH 6.5. The column was eluted with 20 mM phosphate buffer, pH 6.5, at a flow rate of 0.5 mL/min and 3-mL fractions were collected and monitored for protein and OXOAb activity.

Ion exchange chromatography on DEAE-Sepharose. Protein fractions with OXOAb activity collected after gel filtration were loaded onto a DEAE-Sepharose column equilibrated with 20 mM phosphate buffer, pH 6.7. The bound proteins were eluted with a linear gradient of NaCl (0–75%) in 20 mM phosphate buffer, pH 6.7. The 3-mL fractions were collected at the flow rate of 1 mL/min and tested for protein and OXOAb activity. The active fractions were pooled, desalted using ultrafiltration, and stored at −20°C.

Polyacrylamide gel electrophoresis. SDS-PAGE was performed according to the method of Laemmli (1970), using 12% running gel and 5% stacking gel of polyacrylamide. The proteins were silver stained. The molecular weight was calculated using SymGene GeneTools. For the determination of the activity, the SDS was omitted and 9% running gel was prepared. The staining procedure comprised incubation with oxalic acid as a substrate and subsequent incubation with 2,6-dimethoxyphenol and horseradish peroxidase.

RESULTS

Degradation of oxalic acid in A. biennis cultures

In the tested A. biennis cultures, oxalate oxidase (OXOAb) activity was observed only after exogenous addition of oxalic acid as an inductor to the growth media. To confirm the way of oxalic acid degradation by A. biennis, the presence of formic acid in the growth media as a product of decarboxylation of oxalate, and the presence of hydrogen peroxide as a product of oxalic acid oxidation by oxalate oxidase, were tested. As shown in Fig. 1, H₂O₂ was detected in the growth media of A. biennis as the main product of oxalic acid degradation after 24 hours of incubation. This suggests an oxidative activity of A. biennis towards oxalic acid catalysed by oxalate oxidase. Only
in intracellular activity of OXOAb was detected after induction of A. biennis cultures with oxalic acid (Fig. 2). No extracellular oxalate oxidase activity was detected in the growth media. Native intracellular OXOAb was visualised as two protein bands in a polyacrylamide gel, as presented in Fig. 3.

Factors influencing oxalate oxidase production by A. biennis

To specify if the expression of oxalate oxidase in A. biennis mycelium was induced by oxalic acid – the substrate of the enzyme, or by lowering the pH value of the growth media, a solution of oxalic acid, sodium oxalate, and HCl were added separately to the cultures of A. biennis as inducers. As shown in Fig. 4, the activity of A. biennis oxalate oxidase was detected in the mycelium of cultures induced by oxalic acid and HCl, but was not detected in cultures induced by addition of sodium oxalate. This indicates that the lowering of the pH value is sufficient for induction of oxalate oxidase activity during the growth of A. biennis.

Purification of oxalate oxidase from A. biennis

The mycelium of A. biennis from the culture induced with oxalic acid was homogenised and the OXO-specific activity was detected in the supernatant after centrifugation. The supernatant (the crude extract of OXOAb) was loaded onto a Sephadex G-25 column. After chromatography, the OXOAb activity was detected in the first peak (Fig. 5). The next step of the purification procedure was performed on a DEAE-Sepharose column and the elution profile is presented in Fig. 6. Fractions with the high activities of OXOAb were collected and desalted. The enzyme was finally purified 11.1 fold, with 1.7% recovery, when compared to the crude extract (Table 1). After size exclusion and ion-exchange chromatography, the collected fractions exhibiting OXOAb activities were analysed by SDS-PAGE, which revealed a band of protein with a molecular size calculated as 58 kDa (Fig. 7).
Properties of OXOAb

The optimum pH value for OXOAb activity was assayed in the pH range between 2 and 4. OXOAb displayed a maximum activity at pH 2, when incubated at 40°C. This activity decreased with the increasing pH and was almost gone at pH 4 (Fig. 8). The optimum temperature for oxidation of oxalic acid by OXOAb is presented in Fig. 9. When OXOAb was assayed at temperatures in the range from 30°C to 80°C, the highest activity was found at 60°C. To determine substrate specificity, several organic acids, including citric, malic, malonic, glycolic, glioxalic, succinic, and acetic acid, were used as possible substrates for OXOAb. None of the tested carboxylic acids were oxidised by the OXOAb. The effect of the oxalic acid concentration on the initial velocity of the enzyme reaction was tested. The $V_{\text{max}}$ of the enzyme was achieved at 0.03 mol min$^{-1}$. The $K_m$ value for oxalic acid was found to be 0.015 M.

DISCUSSION

In this study, we tested the liquid cultures of A. bienensis to confirm the unique ability of this fungus to degrade oxalic acid by oxidation. In our earlier study, we...
described enhanced oxalate oxidase activity in *A. biennis* in the presence of metal oxides (Graz et al., 2009). Decarboxylation of oxalic acid by oxalate decarboxylase in basidiomycete fungi is typical and well described (Kesarwani et al., 2002; Mäkelä et al., 2002; Mäkelä et al., 2010). The oxidative way of oxalic acid decomposition is atypical for basidiomycete fungi and has been so far described only in *Ceriporiopsis subvermispora* (Aguilar et al., 1999) and *Tilletia controversa* (Vaisey et al., 1961). Oxalic acid can be degraded by oxalate decarboxylase (EC 4.1.1.2) occurring in fungi, by oxalyl-CoA decarboxylase (EC 4.1.1.8) detected in bacteria, and by oxalate oxidase (EC 1.2.3.4) observed in plants (Svedružić et al., 2005). This rigid division of oxalate decomposition activities is now unsettled because of the latest reports concerning fungal oxalate oxidase from *C. subvermispora* (Aguilar et al., 1999) and oxalate oxidase from *A. biennis*, as well as plant oxalyl-CoA decarboxylase found in *Arabidopsis* (Foster et al., 2012). Oxalate oxidase and oxalate decarboxylase are members of the cupin superfamily of proteins. All so far described oxalate oxidases from plants (also known as germins) are classified as monocupins (Dunwell, 2000; Svedružić et al., 2005). The best characterised and purified fungal oxalate oxidase from *C. subvermispora* revealed the oxalate decarboxylase side reaction and was unexpectedly classified as a bicupin protein, the same as all known fungal oxalate decarboxylases (Escutia et al., 2005). The specific activity of purified *C. subvermispora* enzyme expressed in *P. pastoris* showed direct correlation with manganese ions content. Under methanol-induced expression, oxalate oxidase was secreted into the growth media as a soluble and functional enzyme, which is in contrast to the native intracellular enzyme from *C. subvermispora*. The pH optimum was similar and amounted to pH 3.5 for the native protein, and 4.0 for an enzyme expressed in *P. pastoris*. It was suggested that the recombinant protein was inhibited by succinate and by citrate in a competitive and uncompetitive manner, respectively (Moussatche et al., 2011). It was also demonstrated that bacterial oxalate decarboxylase from *B. subtilis* can be converted into an oxidase by mutation in the active site of the enzyme (Burrell et al., 2007). In our study, we have found oxalate oxidase activity (OXOAb) in *A. biennis* mycelium after induction of this enzyme with oxalic acid or HCl. To detect the OXOAb activity in *A. biennis*, the pH dependence of the enzyme was studied. Known fungal oxalate decarboxylases have an inducible character, like an oxalate decarboxylase from basidiomycete *Flammulina velutipes* induced by oxalic acid (Azam et al., 2002), or an enzyme from *Tranetes viridicollor* induced by both, oxalic acid and inorganic acids (Zhu & Hong, 2010). Mäkelä and co-workers (2009) found a rather constitutive expression of an oxalate decarboxylase in *Dichomitus squalens* after addition of oxalic acid to the growth medium. No induction of oxalate decarboxylase activity was observed, but an increase in the transcript amount was observed, which suggests activation rather than transcriptional upregulation of the enzyme. The rapid degradation rate of oxalic acid by *A. biennis* observed in this study could also suggest the activation of the already existing enzyme rather than the induction process, but this hypothesis requires further studies.

All known oxalate degrading enzymes possess optimum activity at low pH values. The OXOAb was active only at acidic pH values below pH 4, with the highest activity at pH 2. A similar optimum pH value (pH 2.6) was described for OXO from *Tilletia controversa* (Vaisey et al., 1961). The oxalate oxidase from *C. subvermispora* showed its maximum activity at pH 3.5, similar to known plant enzymes which exhibited their pH optima in the acidic range varying between pH 3.2, reported for maize roots (Velutić & Sulkić, 2000), pH 3.5 for *Amaranthus spinosus* leaves (Goyal et al., 1999) and wheat seedlings (Hu & Guo, 2009), to pH 5 for *Sorghum* leaves (Pundir & Nath, 1984).

In our study, the optimum temperature for OXOAb activity was 60°C. OXOAb retained 20% of its initial activity after 15 minutes of pre-incubation at 60°C, and only 5% of its initial activity after pre-incubation at 80°C (data not shown). This indicates a protective character of the substrate on the active site of the enzyme. The optimum activity for fungal oxalate oxidase from *C. subvermispora* was obtained at 40°C (Aguilar et al., 1999). Plant enzymes showed their optimum temperatures at 40°C for OXO from *A. spinosus* (Goyal et al., 1999) and 37°C for the enzyme from barley roots (Kotsira & Clonis, 1997). Plant enzymes reveal higher thermal stability, e.g. strawberry fruit oxalate oxidase retained 76% of its initial activity after incubation at 80°C for 30 minutes (Dahiya et al., 2010).

Among the tested organic acids, OXOAb oxidised only oxalic acid. The *Km* value for oxalate was found to be 1.5 × 10⁻² M and the *Vmax* value was 0.03 mol min⁻¹. These parameters are higher than those found for oxalate oxidase from the *C. subvermispora* fungus which has *Km* for oxalate of 1 × 10⁻⁴ M and was inhibited at an oxalic acid concentration above 2.5 mM. Known oxalate oxidases from plants revealed different values of *Km* for oxalate degradation, e.g. it was 1.67 × 10⁻³ M for a strawberry fruit enzyme (Dahiya et al., 2010), 2.1 × 10⁻⁴ M for an enzyme from wheat seedlings (Hu and Guo, 2009), and 2.16 × 10⁻³ M for OXO from *Amaranthus* leaves (Goyal et al., 1999). Oxalate oxidases isolated from plants are multimeric proteins (Svedružić et al., 2005). The molecular weight of fungal oxalate oxidase from *C. subvermispora* was 400 kDa and was suggested to be a homohexameric enzyme. The molecular weight of 58 kDa was observed for OXOAb in this study (SDS-PAGE) corresponds to the molecular weight of a monomer of oxalate oxidase from *C. subvermispora* determined as 65.5 kDa (Aguilar et al., 1999).

**CONCLUSIONS**

Searching for novel enzymes with potentially divergent properties from those described so far seems to be well justified because oxalate-degrading enzymes have a great potential in various biotechnological and diagnostic applications. Oxalate oxidase from *Abortiporus biennis* represents a unique catalytic activity in basidiomycete fungi. In our opinion, development of knowledge in the field of plant and microorganism enzymatic regulation of the level of oxalate in the environment could contribute to progress in the field of bioremediation, geomycology, and agriculture.

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**REFERENCES**


